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STRUCTURAL ORGANIZATION OF THE *CHROMATIUM VINOSUM* REACTION CENTER ASSOCIATED *c*-CYTOCHROMES

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Summary

Magnetic interactions operating between the *Chromatium vinosum* reaction center associated *c*-cytochromes and the electron carriers of the reaction center have been assayed by comparing the magnetic properties of these components alone, and in various combinations with paramagnetic forms of the reaction center electron carriers. These studies have yielded the following results.

1. The oxidized paramagnetic forms of the high potential cytochromes *c*-555 produce no discernable alteration of the light-induced $(BChl)_2^+$ signal.

2. Similarly, analysis of the lineshape of the light-induced $(BChl)_2^+$ signal shows that a magnetic interaction with the oxidized low potential cytochromes *c*-553 is likely to produce less than a 1 gauss splitting of the $(BChl)_2^+$ signal, which corresponds to a minimum separation of 25 ± 3 Å between the unpaired spins if the heme and $(BChl)_2$ are orientated in a coplanar arrangement, suggesting a minimum separation of 15 ± 3 Å between the heme edge and the $(BChl)_2$ edge.

3. A prominent magnetic interaction is observed to operate between the cytochrome *c*-553 and *c*-555, which results in a 30—35 gauss splitting of these spectra, and suggests an iron to iron separation of about 8 Å.

4. Magnetic interactions are not observed between the *c*-cytochromes and the reaction center "primary acceptor" (the iron · quinone complex) nor with the reaction center intermediate electron carrier (which involves bacteriopheophytin) suggesting separations greater than 10 Å.

5. Magnetic interactions are not discerned between the two cytochrome *c*-553 hemes, nor between the two cytochrome *c*-555 hemes, implying that the distance between the cytochromes of the same pair is greater than 10 Å.

6. EPR studies of oriented chromatophores have demonstrated that the cytochrome *c*-553 and *c*-555 hemes are perpendicular to each other, and

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Abbreviation: BChl, bacteriochlorophyll.

suggest that the cytochrome *c*-553 heme plane lies parallel to the plane of the membrane, while the cytochrome *c*-555 heme plane lies perpendicular to the plane of the membrane surface.

Introduction

Light initiated electron transfers from *c*-cytochromes to the reaction center bacteriochlorophyll dimer, $(BChl)_2$, appear to be a general feature of the bacterial photosynthetic apparatus, and provide a convenient opportunity to study the mechanisms and structural parameters leading to cytochrome *c* oxidation [1]. In *Chromatium vinosum* (and several other species of bacteria) two pairs of cytochromes are hydrophobically associated to the reaction center, a pair of high potential cytochrome *c* hemes (cytochrome *c*-555; E_m (pH 7.0) 340 mV [2,3]) and two low potential cytochrome *c* hemes (cytochrome *c*-553; E_m (pH 7.0) 10 mV [2—4]). Remarkably, these four hemes seem to share the same reaction center and compete for the single oxidizing equivalent formed in the light on $(BChl)_2^+$. This cytochrome complement of the *C. vinosum* reaction center has been isolated, and the cytochrome *c*-553 and *c*-555 hemes appear to be contained within a single complex, having a molecular size corresponding to approx. 10 000 daltons per heme [5]. The capacity for the $(BChl)_2$ to react with each of the four cytochrome *c* hemes suggests rather intriguing structural of mechanistic demands.

The two hemes of cytochrome *c*-555 are kinetically (oxidation halftime 2 μs [6—8]) and thermodynamically equivalent at the time $(BChl)_2^+$ is formed [6,9], and a similar equivalence exists with the two cytochrome *c*-553 hemes [4], oxidation halftime 1 μs [7,8]. However, two differences between the electron donation capabilities of the cytochrome *c*-555 and *c*-553 are apparent. Unlike light-induced cytochrome *c*-555 oxidation, which is not significant below temperatures of 200 K [4,10], cytochrome *c*-553 is capable of oxidation down to liquid helium temperatures [11]. The activation energy for this reaction tends to zero below 120 K, where it assumes a halftime of 2.5 ms [11,12]; this reaction has been used as a demonstrable case of electron tunnelling which is relevant to biological electron transfer [11,13]. Secondly, if both cytochromes *c*-553 and *c*-555 are reduced before flash-activation, cytochrome *c*-553 exclusively undergoes oxidation, a phenomenon which is not in keeping with the oxidation rate difference [7,8]. These results have led to suggestions of a possible rapid electron equilibration between the hemes, or structural re-arrangements following cytochrome *c*-553 reduction. The possibility of a direct interaction between the cytochrome *c*-555 and *c*-553 hemes has also been suggested from circular dichroism spectra [5], and may provide a mechanism for the regulation of cytochrome *c*-555 oxidation.

We have studied the magnetic properties of the cytochromes *c* and the $(BChl)_2$ of *C. vinosum* in an attempt to describe the structural organization of the cytochrome complement, and their interactions with the reaction center. We have investigated the magnetic properties of these components from three points of view. The first was to determine if a magnetic coupling exists between the cytochromes *c*-553 and *c*-555 and their common oxidant, $(BChl)_2^+$. The

second was to determine if a magnetic coupling exists between the cytochromes *c*-553 and *c*-555. And thirdly, to determine if these cytochromes are situated close enough to magnetically interact with the remaining electron carriers contained within the reaction center complex: the 'primary' acceptor iron · quinone complex, QFe (see ref. 14 for recent review), and the intermediate electron carrier, I, which acts as the immediate acceptor for the $(BChl)_2$ and donor to the QFe (see ref. 15 for recent review). In addition, we have also presented some preliminary results on EPR studies of orientated chromatophores, which permit determination of the orientation of the cytochrome *c*-553 and *c*-555 hemes with respect to the membrane plane. These results together with the description of the separation of these components yield an outline of the structural parameters involved in the electron transfer reactions between the *C. vinosum* cytochromes *c* and reaction center $(BChl)_2$.

Materials and Methods

Preparative procedures. The photosynthetic bacterium *C. vinosum* was grown anaerobically in the light as described previously [16]. Chromatophores were prepared by passing the cells through a French press followed by the usual differential centrifugation procedures. A subchromatophore particle which contains approx. 30 antenna bacteriochlorophylls per reaction center and associated cytochromes *c* was isolated from chromatophores using the detergent Triton X-100 according to procedures described previously [16]. This preparation has an optical spectrum similar to that reported by Thornber [17] for the subchromatophore "Fraction A" prepared from chromatophores using the detergent sodium dodecyl sulfate. The reaction center · cytochrome complex was isolated from this Triton subchromatophore particle by removal of the antenna bacteriochlorophyll and major portion of carotenoids [16].

Spectrometry. Electron paramagnetic resonance (EPR) spectra were recorded with either a Varian E109 or E4 spectrometer, equipped with a flowing helium cryostat and temperature control.

Redox potentiometry. The apparatus used for anaerobic redox poising and transfer of samples of EPR tubes for low temperature analysis has previously been described [4,18].

Generation of the C. vinosum reaction center redox states. Table I presents an outline of the experiments and a summary of the various redox states which have been used to study the magnetic interactions operating between the electron carriers of the *C. vinosum* reaction center · cytochrome complex. The systematic generation of different combinations of paramagnetic species of *C. vinosum* has been accomplished by making use of basic redox potentiometry and the well-known light-activated reactions of *C. vinosum* at low temperature. The experimental conditions and the redox states of the system before and after illumination are also summarized in Table I, and are described with more detail in the references given in the footnotes. In this table, and throughout the paper, redox potentials listed represent the values established at room temperature, prior to rapid freezing in the dark and storage in liquid nitrogen.

TABLE I

C. VINOSUM REACTION CENTER · CYTOCHROME COMPLEX REDOX STATES

Redox potential (E_h)	+550 mV	+420 mV	+200 mV	−60 mV	−250 mV
Dark redox states	(1) c^+-553 c^+-553 c^+-555 $[(BChl)_2^+ I]QFe$ c^+-555	(2) c^+-553 c^+-553 c^+-555 $[(BChl)_2 I]QFe$ c^+-555	(4) c^+-553 c^+-553 c-555 $[(BChl)_2 I]QFe$ c-555	(6) c-553 c-553 c-555 $[(BChl)_2 I]QFe$ c-555	(9) c-553 c-553 c-555 $[(BChl)_2]\ Q^{\cdot-}Fe$ c-555
	$h\nu$ ↓	b ⇅ a $h\nu \lesssim 300$ K	b,c ⇅ a $h\nu < 200$ K	a,d ↓ $h\nu < 200$ K	a,e ⇅ $h\nu < 80$ K
Light-generated redox states	No change	(3) c^+-553 c^+-553 c^+-555 $[(BChl)_2^+ I]Q^{\cdot-}Fe$ c^+-555	(5) c^+-553 c^+-553 c-555 $[(BChl)_2^+ I]Q^{\cdot-}Fe$ c-555	(7) c^+-553 c-553 c-555 $[(BChl)_2 I]Q^{\cdot-}Fe$ c-555	(10) c-553 c-553 c-555 $[(^+BChl^{\cdot-})_2 I]Q^{\cdot-}Fe$ c-555
				a,e ⇅ $h\nu < 80$ K	a,f ↓ $h\nu < 200$ K
				(8) c^+-553 c-553 c-555 $[(^+BChl^{\cdot-})_2 I]Q^{\cdot-}Fe$ c-555	(11) c^+-553 c-553 c-555 $[(BChl)_2 I^{\cdot-}]Q^{\cdot-}Fe$ c-555

[a] The initial reaction observed following the absorption of a quantum of light, which is well studied in *Rps. sphaeroides*, is the oxidation of $(BChl)_2$ and the reduction of I, forming $(BChl)_2^+ I^-$ in less than 10 ps [45—48]. If the QFe is oxidized, then I^- appears to reduce the QFe complex with a halftime of 100—200 ps [45—48].

[b] The back reaction from Q^-Fe to $(BChl)_2^+$ below 80 K shows a 20—25 ms halftime in both *C. vinosum* and *Rps. sphaeroides* chromatophores [12,19,49]. In *Rps. sphaeroides* this rate is found to be temperature sensitive above 120 K, and has a 60 ms halftime at 300 K [50].

[c] At redox potentials such that the cytochromes *c*-553 are poised oxidized and the cytochromes *c*-555 are poised reduced (300 mV > E_h > 100 mV), the light generated $(BChl)_2^+$ will be rapidly reduced at room temperature by the cytochromes *c*-555 with a 2 μs halftime [6—8]. At temperatures below 200 K this reaction is prevented, permitting the generation of $(BChl)_2^+$ on a steady state basis [4,10,51].

[d] The reduced cytochromes *c*-553 will reduce the light generated $(BChl)_2^+$ with a 1-μs halftime 300 K, which slows to 2.5 ms at temperatures below 120 K [11,12]. At temperatures below 200 K this light-induced cytochrome oxidation is essentially irreversible [4,51], and traps an electron on QFe.

[e] If the QFe is reduced before light activation, either by chemical reduction by poising at redox potentials below −200 mV or by light generation in *C. vinosum* as decribed above, then the normal forward electron transfer from I^- to QFe is blocked, and the electron returns to $(BChl)_2^+$ [14,15,24,25]. In *Rps. sphaeroides* this occurs with a temperature-dependent halftime of 10 ns at 300 K, and 30 ns below 80 K [52]. At temperatures below 80 K, this return of an electron to $(BChl)_2^+$ forms the $(BChl)_2$ triplet/biradical [14,15,52], and is observed by EPR as the spin polarized triplet signal [24,25]. In *Rps. sphaeroides* the decay of this $(BChl)_2$ triplet/biradical state occurs a 6 μs halftime at 300 K and 120 μs halftime between 120 and 10 K [52].

[f] In *C. vinosum* under conditions in which the normal forward photochemistry is blocked, cytochrome *c*-553 donation to the light generated $(BChl)_2^+$ competes with the return of an electron from I^-, and oxidation of the cytochrome under these conditions generates the trapped I^- redox state [16]. At 200 K the cytochrome oxidation halftime of 10 s [11,12] competes with the approx. 10 ns oxidation of I^- [52], while below 120 K the cytochrome reaction slows to 2.5 ms [11,12]. The irreversibility of the cytochrome reaction permits accumulation of the trapped I^- state following prolonged illumination [16].

Calculation of distances. The distances between unpaired spins located on the electron carriers of the reaction center-cytochrome complex were calculated by assuming that alteration of the EPR spectra arose from magnetic dipolar interactions between the unpaired spins. The magnitude of the magnetic interactions were estimated by calculation of the amount of dipolar splitting required to produce the modified EPR spectra, and these splittings were substituted into the standard magnetic dipolar equation to calculate the separation between non-identical spins (20):

$$\Delta H = g\beta(1 - 3\cos^2\theta)/r^3$$

Here, ΔH represents the dipolar splitting; g is the electron spin g-factor for the accompanying paramagnetic species; β is the Bohr magneton; θ is the angle between the line connecting the two paramagnetic centers and the magnetic field; r is the distance between unpaired spins.

In addition to a magnetic dipolar interaction, contributions to the splitting of an EPR spectrum may also arise from a magnetic exchange coupling between unpaired spins, which requires an overlap of the electron orbitals [20]. This overlap could either arise from a direct "contact" between interacting molecules, or through a bridging molecule [20].

Results

The low potential cytochromes c-553 and interactions with I and QFe

The pair of low potential cytochromes *c*-553 can be studied in the oxidized, paramagnetic form, while the pair of high potential cytochromes *c*-555 and the reaction center electron carriers are in diamagnetic redox states. This is done simply by poising the *C. vinosum* preparations at redox potentials near 200 mV before freezing (Table I, redox state 4). Fig. 1 shows the EPR spectrum for the reaction center · cytochrome complex poised in this redox state, and shows the characteristic cytochrome *c*-553 EPR adsorption which can be identified by the $g_z = 2.93$ and $g_y = 2.24$ peaks [19]; in addition a trough, not previously reported, is also observed at $g = 1.54$ which has the same redox dependence and low temperature light-induced oxidation properties as the $g = 2.93$ and $g = 2.24$ signals, and can be identified as the cytochrome *c*-553 g_x absorption.

Fig. 2C shows the details of the g 3 region, which permits a measurement of the width at half height to be 56 gauss in the reaction center complex for the ferricytochromes *c*-553 g_z absorption. In the Triton subchromatophore 'Fraction A' [17], this absorption is observed to have about 45 gauss width at half height (Fig. 3C). The microwave power saturation profile for the oxidized cytochromes *c*-553 poised in this redox state is shown in Fig. 4B, and is essentially identical when measured either in the reaction center · cytochrome complex or in Fraction A.

Also apparent in Figs. 1—3 are unidentified paramagnetic species having EPR absorptions centered at $g = 3.4$, 4.3, and 5.9 [19]; optical spectra associated with these components have not been identified in the reaction center · cytochrome complex.

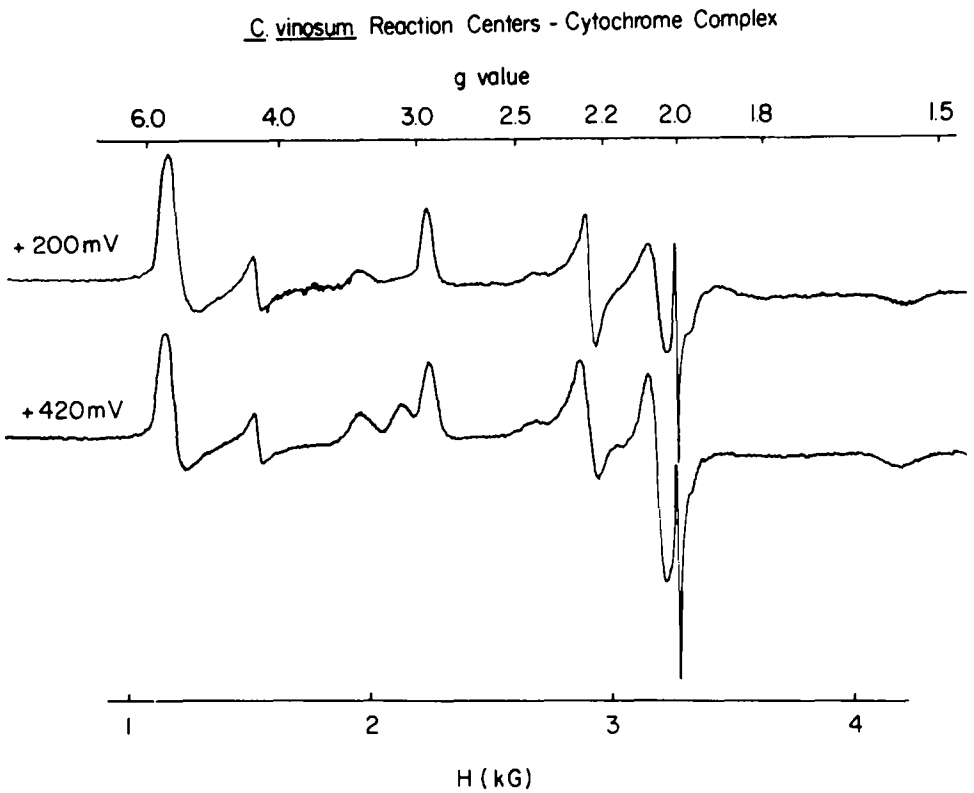

Fig. 1. EPR spectra of the *C. vinosum* reaction center · cytochrome complex. The reaction center · cytochrome complex (A_{883nm}, 5.0 cm^{-1}) was suspended in 0.1% Triton X-100, 20 mM Tris—HCl, pH 8.0, and poised at redox potentials of either 200 or 420 mV before freezing. Spectra were recorded at 10 K using 1 mW of microwave power, 25 gauss modulation amplitude and a spectrometer gain of $6.3 \cdot 10^3$.

By redox poising the reaction center · cytochrome such that both cytochromes *c*-553 are reduced (redox state 6), and by taking advantage of the ability for the light-induced $(BChl)_2^+$ to oxidize irreversibly either cytochrome of this pair at low temperature [4,11,12], it is possible to generate one ferricytochrome *c*-553 heme, while both the cytochromes *c*-555 and the companion cytochrome *c*-553 remain in diamagnetic, reduced forms. Depending upon the redox state of the QFe complex before the low temperature, light-induced oxidation, the ferricytochrome *c*-553 hemes can be formed either in the presence of Q^-Fe and I^- or in the presence of Q^-Fe alone (Table I, redox states 11 and 7). Detailed spectra in the $g = 3$ region are shown respectively in Fig. 2A and B for the reaction center · cytochrome complex in redox states 11 and 7, and similarly in Fig. 3A and B for the Fraction A. In both redox states the single ferricytochrome *c*-553 g_z absorbance is shifted by 6—10 gauss to lower field values, and the widths are 3—5 gauss narrower than those observed when both hemes are oxidized; in contrast, the lineshape and positions for the g_x and g_y components, however, are observed not to change. The microwave power saturation profiles for the ferricytochrome *c*-553 in all three redox states (i.e., redox state 4, 7 and 11) are essentially identical. The lack of

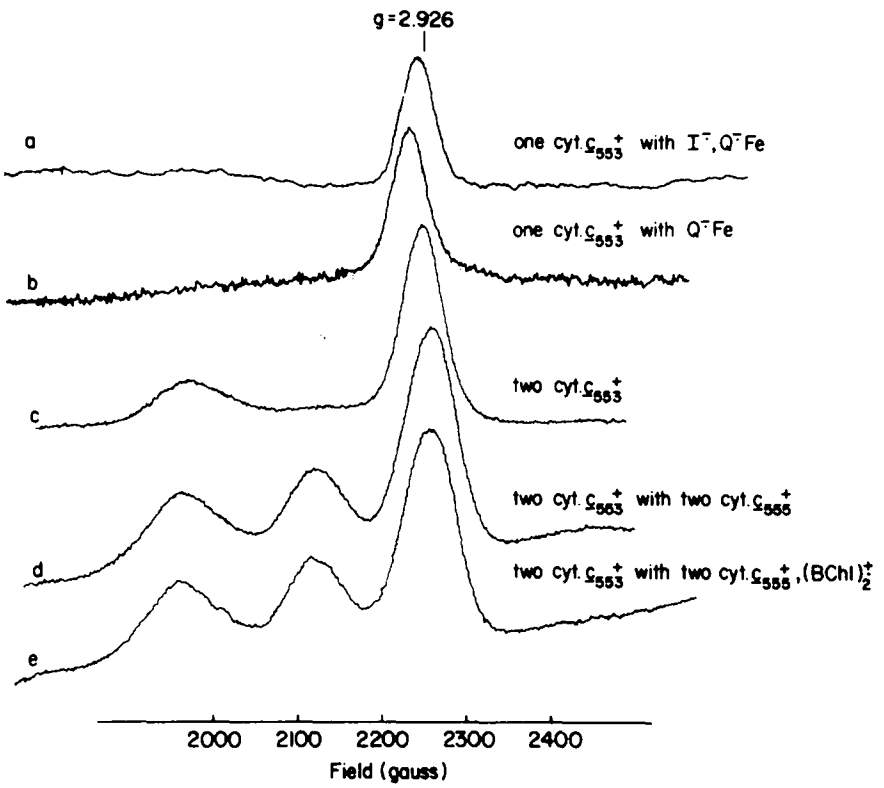

Fig. 2. EPR spectra of the $g = 3$ region for the various redox states of the *C. vinosum* reaction center · cytochrome complex. Redox states were generated as described in Table I. Spectra a through e correspond to redox states 11, 7, 4, 2, 1, respectively. The spectrometer gain for parts a and b was $10 \cdot 10^3$, and $6.3 \cdot 10^3$ for parts c through e. Other conditions were as described in Fig. 1.

line broadening and the absence of an increased rate of spin relaxation in the singly oxidized cytochrome *c*-553 redox states, which are formed in the presence of the more rapidly relaxing I^{-} and Q^{-}Fe species, indicate that these hemes are magnetically isolated from both the I^{-} and Q^{-}Fe reaction center components. The absence of observable dipolar interactions suggests that the distances are greater than 10 Å.

Similarly, the lack of line-broadening of the g_y and g_x absorptions of the cytochromes *c*-553 upon changing from a single to both cytochromes *c*-553 oxidized shows that magnetic interaction between the two cytochrome *c*-553 hemes are not of sufficient strength to alter the observed spectra. The broadness of the EPR lines would prevent observation of a magnetic dipolar interaction unless these hemes were closer than 11—12 Å. However, alteration in the precise position and width of the cytochrome *c*-553 g_z peak may reflect non-magnetic, possibly electrostatic, interactions between the two cytochrome *c*-553 hemes, or between these cytochromes I^{-} or Q^{-}Fe (see Discussion).

It is also noteworthy that the unidentified $g = 3.4$, 4.2 and 5.9 species are not evident in samples which have been poised at redox potentials below 0 mV. The constancy of the spin relaxation rate of the ferricytochrome gene-

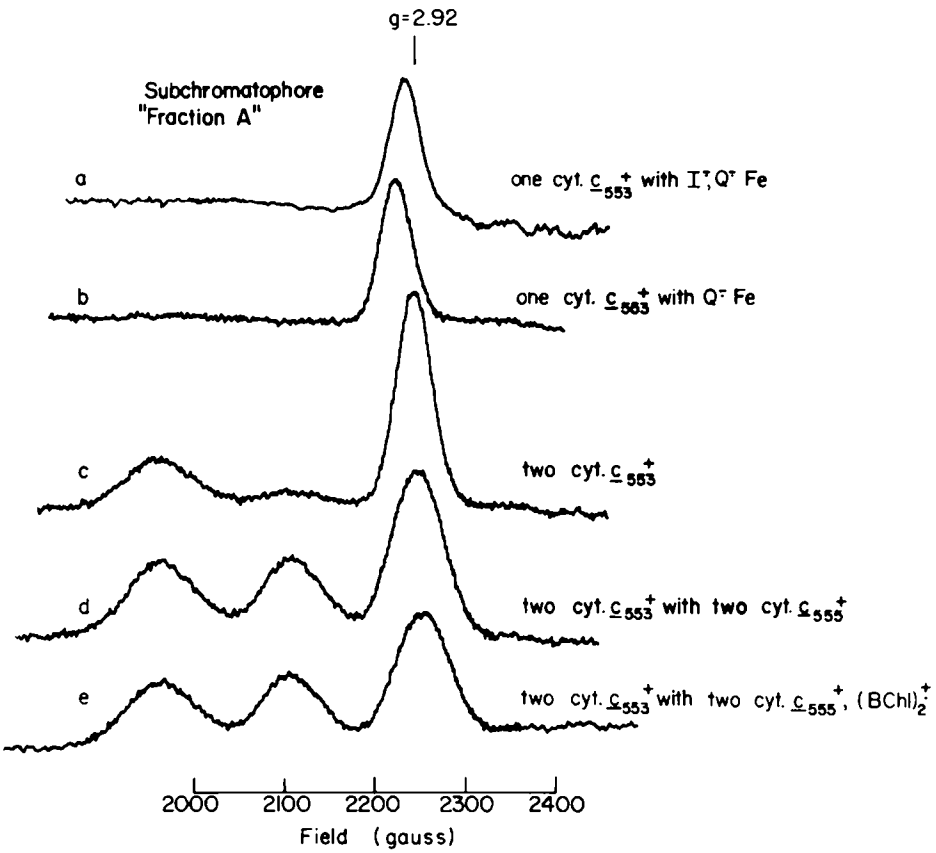

Fig. 3. EPR spectra of the $g = 3$ region for the *C. vinosum* subchromatophore Fraction A. The various redox states were generated with conditions as described in Table I. Spectra a through e correspond to redox states 11, 7, 4, 2, 1, respectively. The subchromatophore particles were suspended in 0.1% Triton X-100 20 mM Tris, pH 8.0, with a bacteriochlorophyll concentration of 1.5 mM. The spectrometer gain for spectra a and b was $1.6 \cdot 10^4$ and $1.25 \cdot 10^4$ for spectra c through e, with a 16 gauss modulation amplitude and 1 mW microwave power in each case. The spectra were recorded at 11 K.

rated under redox conditions above and below 0 mV suggests that no interaction exists between cytochrome *c*-553 and these unknown species.

The high potential cytochromes c-555 and interactions with I and QFe

Poising at a redox potential of 420 mV causes a greater than 95% oxidation of the high potential cytochromes *c*-555, while the $(BChl)_2$ remains greater than 90% reduced (Table I, redox state 2). Fig. 1 also shows the EPR spectrum for the reaction center · cytochrome complex poised in this redox state, and shows the characteristic cytochromes *c*-555 $g_z = 3.1$ absorption [19]; the g_y and g_x components of this spectrum have not yet been identified.

In contrast to the ability to generate singly oxidized cytochrome *c*-553 redox states, we have not succeeded in generating a single oxidized cytochrome *c*-555 in the presence of Q^-Fe or I^-, so that magnetic interactions between the two cytochrome *c*-555 hemes can not be assayed in this manner. However, it is possible to look for interactions between the cytochrome *c*-555 hemes by

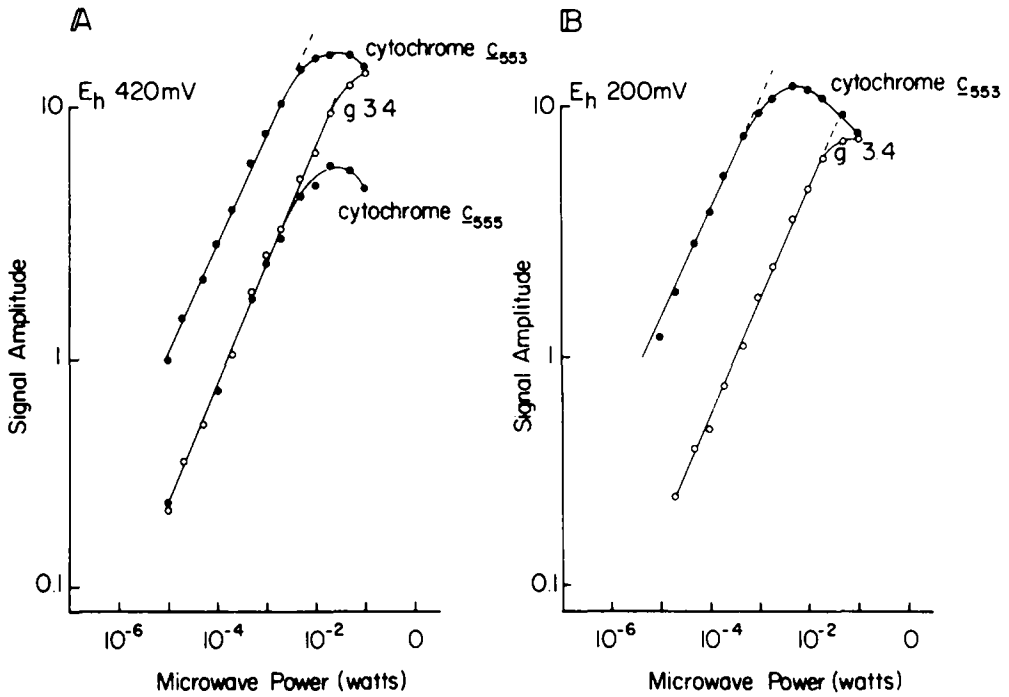

Fig. 4. Microwave power saturation profiles for the *C. vinosum* cytochromes *c*. The saturation profiles were obtained for the components of the reaction center · cytochrome complex having an absorbance in the $g = 3$ region in samples previously poised at redox potentials of 420 mV (Part a), and 200 mV (Part b). Conditions were those described in Fig. 2 for spectra c and d.

comparing the EPR spectrum of partially and fully oxidized cytochrome *c*-555. In samples poised at redox potentials below the midpoint, the majority of reaction centers will have only one cytochrome oxidized, while at much higher redox potentials all reaction centers will have two oxidized cytochrome *c*-555 hemes. Although the proximity of the unidentified $g = 3.4$ signal and cytochrome *c*-553 signals make measurements difficult in the partially oxidized samples when the cytochrome *c*-555 signal is relatively small, the lineshape of the 78—80 gauss wide cytochrome *c*-555 $g = 3.1$ signal is observed not to change through the course of the redox titration shown in Fig. 5A. These results suggest that magnetic interactions between the two cytochrome *c*-555 hemes are not large enough to be observed in the EPR signal, which requires a separation greater than 8—10 Å.

The absence of an alteration of the spin relaxation properties of the cytochromes *c*-555 in the presence of the light-induced Q^-Fe (Table I, redox state 3) indicates that these hemes, like the cytochromes *c*-553, are likely to be separated by a large distance from the QFe. The possibility of a magnetic interaction between these cytochromes and the more slowly relaxing $(BChl)_2^+$ is examined in detail below.

Interaction between the cytochrome c-553 and c-555 pairs

Oxidation of the cytochrome *c*-555 hemes (Table I, redox state 2) is seen to

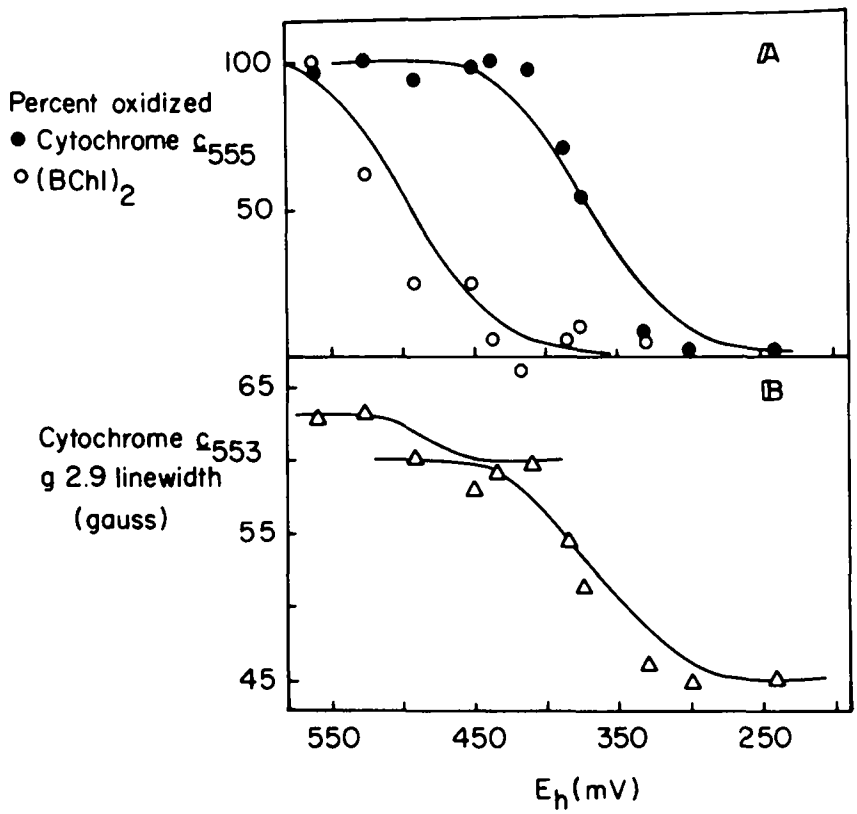

Fig. 5. Redox potential dependencies of the cytochrome *c*-555, $(BChl)_2$ EPR signals and the linewidth of the ferricytochrome *c*-553 EPR signal, measured in the *C. vinosum* subchromatophore Fraction A. In (A) the extent of cytochrome *c*-555 oxidation was measured by the relative amplitude of the $g = 3.1$ signal, and the extent of $(BChl)_2$ oxidation was measured by subtraction of the extent of the light-induced $g = 2$ signal seen in a given sample from that of the maximum light-induced signal seen in samples poised at redox potentials near 200 mV. (B) shows the redox potential dependence of the linewidth measured at half-height of the cytochrome *c*-553 $g = 2.93$ derivative absorbance. Other conditions are listed in Fig. 3.

alter EPR spectrum of the cytochromes *c*-553. A detailed spectrum of the $g = 3$ region (Fig. 2D) reveals the cytochromes *c*-553 g_z linewidth at half height to have broadened by about 16 gauss to about 72 gauss, and shows the peak absorption to have shifted to a slightly higher magnetic field. A close inspection of Fig. 1 also indicates that the 45 gauss wide cytochrome *c*-553 g_y absorbance has also broadened by about 35 gauss upon oxidation of the cytochromes *c*-555. The possibility that part of this broadening arises from a result of an appearance of a broader, overlapping cytochrome *c*-555 g_y absorbance cannot be ruled out. Similarly, in Fraction A the cytochrome *c*-553 g_z absorption is also seen to broaden by 15—20 gauss from about 45 gauss to 60—65 gauss (Fig. 3D), while the 45 gauss wide g_y component also broadens by about 35 gauss. *K*-band EPR spectra would be helpful toward separating these resonance assessing any accompanying interactions.

Fig. 5 shows the redox potential dependence for the linewidth of the cytochrome *c*-553 g_z absorption measured in the subchromatophore Fraction A, and this is plotted with the extent of cytochrome *c*-555 oxidation, measured

by the $g = 3.1$ absorption. These results identify a close correlation between the linewidth of the cytochromes *c*-553 EPR spectrum and the oxidation state of the cytochromes *c*-555. In addition to alteration of the cytochrome *c*-553 EPR spectrum, the oxidized cytochromes *c*-555 also enhance the spin relaxation rate of the cytochrome *c*-553 (Fig. 4A). The possibility that ferricyanide, which was used as an oxidant in these titrations, acted as a magnetic perturbant was ruled out by oxidizing cytochrome *c*-555 in a different way. Cytochrome *c*-555 was oxidized in the absence of added oxidants by a 1 min period of illumination at room temperature followed by rapid freezing in liquid nitrogen in the dark. This procedure results in virtually complete oxidation of the cytochrome *c*-555 complement while $(BChl)_2$ remains reduced [19], and yields essentially identical changes in the EPR spectrum of cytochrome *c*-553 as those seen by chemical oxidation of cytochrome *c*-555.

While these changes in the magnetic properties of the cytochrome *c*-553 demonstrate the existence of an interaction between the cytochrome *c*-553

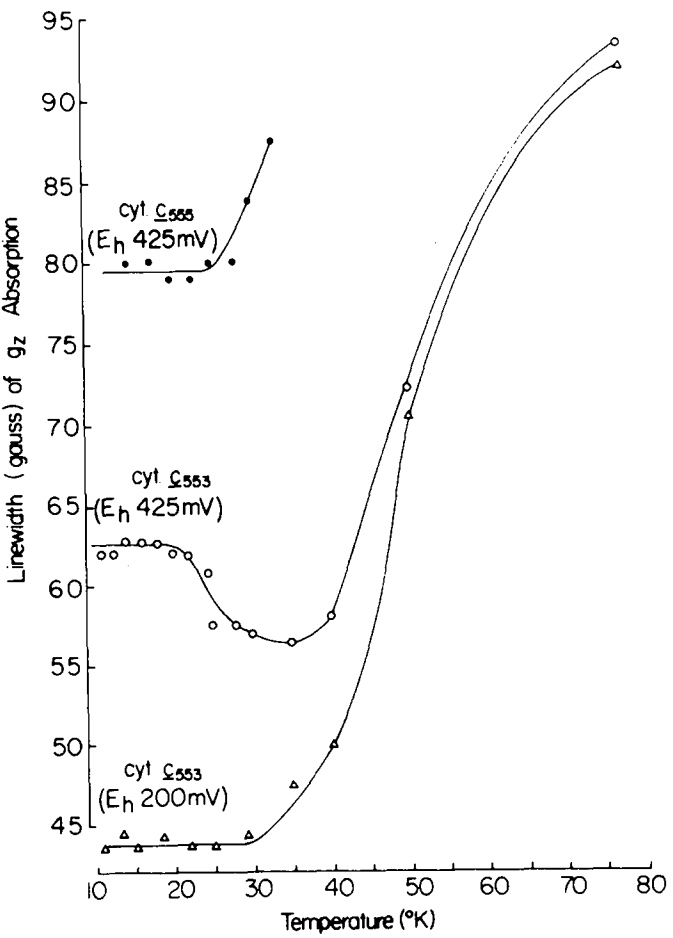

Fig. 6. Temperature dependence of the linewidth of the cytochrome g_z peaks measured in the subchromatophore Fraction A. The width at half-height of the cytochrome *c*-553 g_z absorption was measured as a function of temperature in both redox states 2 and 4, with conditions as described in Fig. 3.

and c-555 hemes, it is necessary to distinguish between a heme interaction mediated by protein conformation changes or a direct magnetic interaction. We have attempted to distinguish between these two possibilities by studying the temperature dependence of the cytochrome c-553 linewidth.

Fig. 6 shows the temperature dependence of the linewidth for the cytochrome c-553 g_z peak, measured in the subchromatophore Fraction A. For the oxidized cytochrome c-553 spectrum measured in the presence of reduced cytochrome c-555, these results show that line-broadening occurs at temperatures above 30 K, which can be attributed to an increasing spin lattice relaxation rate and subsequent lifetime/broadening effects [20]. The altered cytochrome c-553 spectrum produced by the oxidation of the cytochrome c-555 hemes reveals however, a narrowing at temperatures above 20 K, which continues until approx. 30 K, and then begins to broaden. The linewidths of the cytochrome c-553 spectra measured in both redox states become essentially equivalent above 50 K.

The narrowing of the cytochrome c-553 spectrum at the intermediate temperatures is expected if the broadening of the signal at low temperatures in the presence of oxidized c-555 represents an unresolved splitting arising from a magnetic interaction. A collapse of the splitting, and narrowing of the cytochrome c-553 signal, should occur as the magnitude of the spin relaxation rate of cytochrome c-555 exceeds the magnitude of the splitting [20]. This kind of temperature-dependent broadening would be difficult to explain if the broadening of the cytochrome c-553 spectrum arose from protein mediated conformation changes accompanying cytochrome c-555 oxidation, and may serve to identify a direct magnetic interaction between the cytochrome c-553 and c-555 hemes.

A 30—35 gauss splitting of the cytochrome c-553 g_z absorption can account for the observed linebroadening in each preparation, and could arise from either a magnetic exchange or dipolar interaction. An exchange interaction would suggest an electron orbital overlap [20] and a close association between the cytochromes c-553 and c-555, which would be expected to permit a rapid electron transfer between these hemes. Electron transfer between the cytochromes c-553 and c-555 has not been reported, which might tend to rule out the possibility of an electron exchange mechanism operating between the cytochrome c-553 and c-555 hemes.

A magnetic dipolar interaction of 30—35 gauss corresponds to a separation of approx. 8 Å between unpaired spins, and can be interpreted to represent the iron to iron separation between the cytochrome c-553 and c-555 hemes. The increased broadening of the g_y component of the cytochrome c-553 spectrum is compatible with a perpendicular arrangement of the cytochrome c-553 and c-555 heme planes, in which the cytochrome c-555 g_z direction approaches a parallel orientation with the cytochrome c-553 g_y direction. However, confirmation of these speculations would require the identification of the cytochrome c-555 g_x and g_y absorption peaks.

Interaction between the c-cytochromes and the $(BChl)_2$

At redox potentials high enough to oxidize $(BChl)_2$ (Table I, redox state 1) cytochrome c-553 spectra appear to exhibit a slight additional increase in

width (Figs. 2E and 3E). Such a slight alteration of the respective 72 and 65 gauss wide lines is at the limit of resolution, but would require, however, an approximate 10—15 gauss splitting of these spectra if these broadenings arose from a magnetic interaction with the $(BChl)_2^+$. The approx. 10 gauss wide $(BChl)_2^+$ signal certainly does not reveal an interaction of this magnitude, and suggests that these linebroadenings are likely to arise as a result of the high redox potentials required to oxidize the $(BChl)_2$. As a result, the relatively broad cytochrome spectra do not permit the resolution of weak magnetic interactions operating between the *c*-cytochromes and the $(BChl)_2$.

Instead, a magnetic interaction between $(BChl)_2$ and cytochrome *c*-555 can be conveniently assayed by comparing the lineshape of the light induced $(BChl)_2^+$ signal formed in the presence of reduced or oxidized cytochrome *c*-555 (Table I, redox states 3 and 5) at temperatures below 20 K. No alteration is detected in the light induced $(BChl)_2^+$ signal upon oxidation of the cytochrome *c*-555, and a peak to peak derivative linewidth of 10.4 ± 0.1 gauss is measured in both redox states.

Owing to the rapid (2 ms) low temperature electron transfer reaction

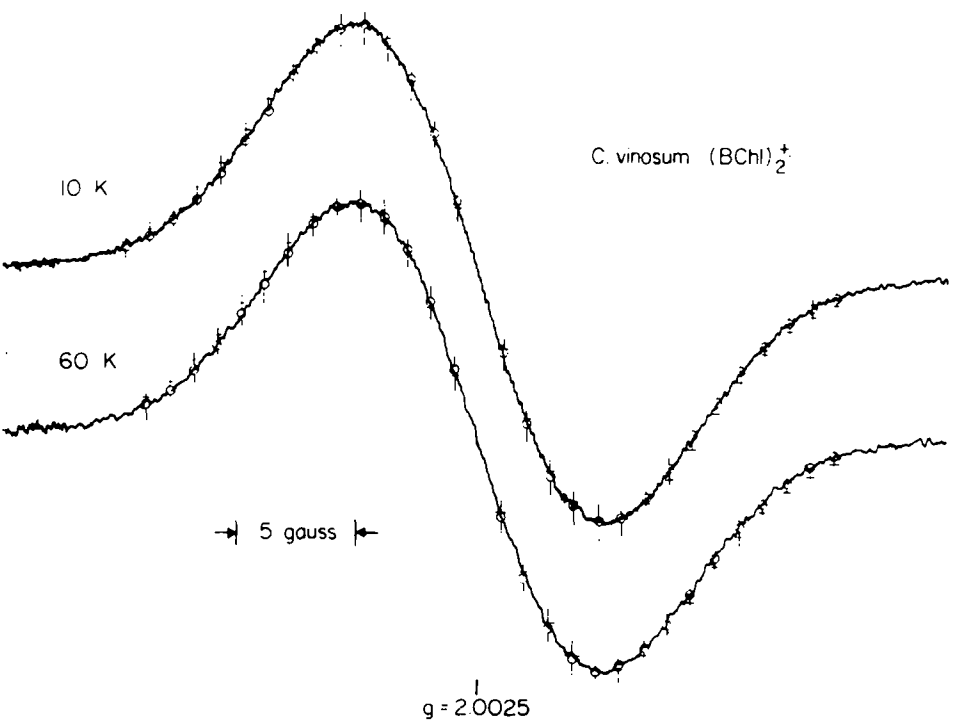

Fig. 7. The ligh-induced $(BChl)_2^+$ signal measured in the presence of oxidized cytochrome *c*-553 and reduced cytochrome *c*-555 (Table I, redox state 5) in the subchromatophore Fraction A. Spectra were recorded at 60 and 10 K with spectrometer gains of $2 \cdot 10^4$ and $1 \cdot 10^4$ and microwave powers of 0.1 mW and 0.01 W respectively, using a modulation amplitude of 3.2 gauss in each case. Other conditions are as described in Fig. 2. The superimposed circles and crosses are points calculated for gaussian derivative lineshapes. For the 60 K spectrum the circles are derived from a gaussian line having a 10.2 gauss peak to peak linewidth; the crosses are generated from a gaussian line having a 10.5 gauss peak to peak linewidth. For the 10 K spectrum the circles correspond to a lineshape resulting from a 2 gauss splitting of the 10.2 gauss wide line; the crosses from a 3 gauss splitting of the 10.2 gauss wide line.

between reduced cytochrome *c*-553 and $(BChl)_2^+$, interactions between this cytochrome and $(BChl)_2$ cannot easily be assayed by the above method. However, by taking advantage of the rapid spin lattice relaxation rate of the ferricytochrome *c*-553 at temperatures above 30 K, the existence of a magnetic interaction between cytochrome *c*-553 and $(BChl)_2$ can be assayed by comparing the lineshape of the light induced $(BChl)_2^+$ signal (Table I, redox state 5) at temperatures above and below 30 K. Fig. 7 shows the EPR spectra of the light induced $(BChl)_2^+$ signal recorded at 10 and 60 K. By fitting the spectra with gaussian-shaped derivative curves it was found that the spectrum recorded at 60 K could be matched by a gaussian curve having a peak to peak linewidth of 10.2 ± 0.1 gauss, as shown by the circles in Fig. 7. For comparison, the crosses show the lineshape corresponding to a 10.5 gauss wide gaussian line. Curve fitting to the 10 K spectrum showed that this signal could be matched with a curve having a linewidth of 10.4 ± 0.1 gauss. If this apparent broadening were caused by an unresolved splitting of the 10.2 gauss wide line, it would correspond to a splitting of 2 gauss as shown by the circles in Fig. 7 and could possibly reflect a magnetic coupling with the cytochromes *c*-553 having a 22 Å separation. The crosses indicate the poor fit to the lineshape arising from an assumed 3 gauss splitting of a 10.2 gauss wide gaussian line.

However, the linewidth of the EPR spectrum for the bacteriochlorophyll cation radical in vitro also shows a variation with temperature, which changes from a derivative peak to peak width of 13.5 gauss at 80 K to 14 gauss at 2 K [21], and has been assigned to result from a freezing out of the motion of methyl groups [21]. Similar motions apparently also influence the line-width of the in vivo $(BChl)_2^+$ [21]. The $(BChl)_2^+$ signal in partially deuterated *Rhodopseudomonas sphaeroides* R-26 chromatophores and isolated reaction centers, which are free of associated *c*-cytochromes, has a linewidth of about 5.5 gauss at 80 K, which broadens to 6.8 gauss at 1.4 K (Isaccson, R. and Feher, G., personal communication). With fully protonated *Rps. sphaeroides* R-26 reaction centers we have measured a linewidth of 9.7 ± 0.1 gauss for the light-induced $(BChl)_2^+$ signal at 10 K, and a 9.5 ± 0.1 gauss linewidth at 60 K. These observations provide an alternative and perhaps more likely explanation for the linewidth changes observed for the *C. vinosum* $(BChl)_2^+$. If similar nuclear hyperfine interactions and temperature dependent motion of the methyl groups exist in the *C. vinosum* $(BChl)_2^+$ (which could be confirmed by an investigation of the electron nuclear double resonance (ENDOR) transitions in *C. vinosum*), then these results would suggest that magnetic interactions between the cytochrome *c*-553 and $(BChl)_2$ are not responsible for the temperature-dependent changes in the *C. vinosum* $(BChl)_2^+$ signal, and would require the lack of an observable magnetic coupling between the cytochrome *c*-553 and $(BChl)_2$.

An upper limit on the strength of a magnetic interaction between cytochrome *c*-553 and $(BChl)_2$ can then be placed as less than 1 gauss, which would cause just under 0.1 gauss of broadening of the $(BChl)_2^+$ signal. A maximum magnetic dipolar interaction of 1 gauss would correspond to a minimum separation of about 27 Å between the unpaired spin on the cytochrome *c*-553 and $(BChl)_2$, assuming a coplanar arrangement, and would suggest a separation of about 17 Å between the heme and $(BChl)_2$ edges, using an effective radius

of 5 Å for the heme and $(BChl)_2$. The appropriateness of the point dipole approximation for this situation arises from the fact that the unpaired electron spin for the cytochrome *c*-553 is likely to be localized primarily on the iron, as shown for mammalian cytochrome *c* [22,23], and from the relatively large separation between the $(BChl)_2$ and the heme, which minimizes the effect of electron delocalization in $(BChl)_2$, and suggests that these calculations represent good estimates of the actual separation.

A potential interaction between the cytochrome *c*-553 and $(BChl)_2$ can also be assayed by a comparison of the lineshape of the light induced $(BChl)_2$ triplet/biradical signal [24,25], formed in the presence of a single oxidized cytochrome *c*-553 heme, or fully reduced cytochrome *c*-553 (Table I, redox states 8 and 10). With samples poised before freezing at a redox potential of —60 mV, we have generated the oxidized cytochrome *c*-553 heme and reduced QFe by illumination at 7 K. At this temperature repositioning of the heme following oxidation is not likely to occur, and the distance determined for the separation of the cytochrome *c*-553 heme and the $(BChl)_2$ must represent the actual distance of electron transfer.

We observe no alteration in lineshape or linewidth of the triplet/biradical

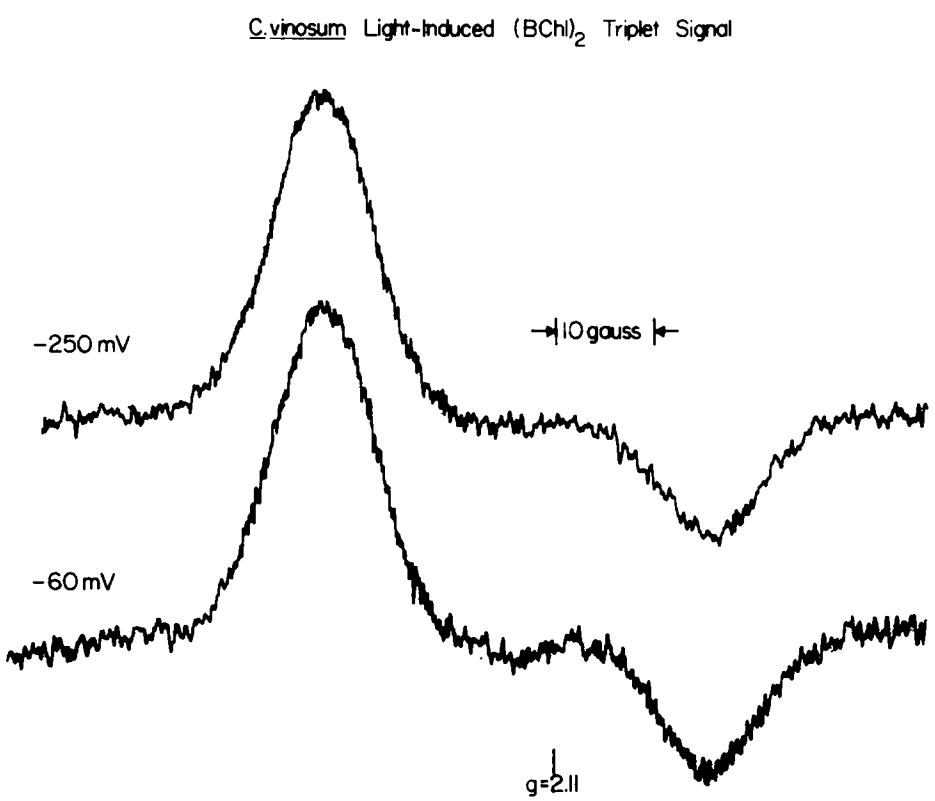

Fig. 8. The light-induced $(BChl)_2$ triplet/biradical signals recorded in the presence of fully reduced and singly oxidized cytochrome *c*-553 in the subchromatophore Fraction A. Samples were poised in redox states 10 and 8 as described in Table I, and the light-induced $(BChl)_2$ triplet/biradical signals were recorded at 6 K using microwave power levels of 0.5 mW and an 8 gauss modulation amplitude with spectrometer gains of $5 \cdot 10^4$ and $6.3 \cdot 10^4$, respectively. Other conditions were as described in Fig. 3.

signal accompanying the oxidized cytochrome *c*-553. Repeated recording of the $(BChl)_2$ triplet/biradical signal in the presence of reduced and singly oxidized cytochrome *c*-553 consistently yields a measurement of 12 gauss for the full width at half height of low field absorption and emission peaks. Fig. 8 shows representative spectra of the $(BChl)_2$ triplet/biradical signal recorded in each redox state. These results again demonstrate the absence of a prominent magnetic interaction operating between the cytochrome *c*-553 and $(BChl)_2$, and suggest that the oxidized cytochrome *c*-553 hemes produces less than 0.5 gauss broadening of the $(BChl)_2$ triplet/biradical spectrum, which would correspond to an unresolved splitting of less than 3 gauss.

Orientation of the cytochrome c hemes

X-ray diffraction [26] and linear dichroism [27] studies have shown that drying of a suspension of chromatophores results in the formation of well oriented membrane multilayers. Similarly, the formation of oriented multilayers has also been demonstrated with artificial phospholipid vesicles, including vesicles containing bacterial photosynthetic reaction centers [28], or cytochrome oxidase [29], and has permitted the determination of the orientation of the hemes in cytochrome oxidase [29,30]. We have used this technique to study the orientation of the electron transfer components in the *C. vinosum* chromatophore, and we present here some preliminary results on the orientation of the cytochromes *c*-553 and *c*-555 as determined by EPR.

Washing chromatophores with 1 mM ferricyanide in 10 mM MOPS, pH 7.0 followed by drying, results in the formation of oriented samples having both

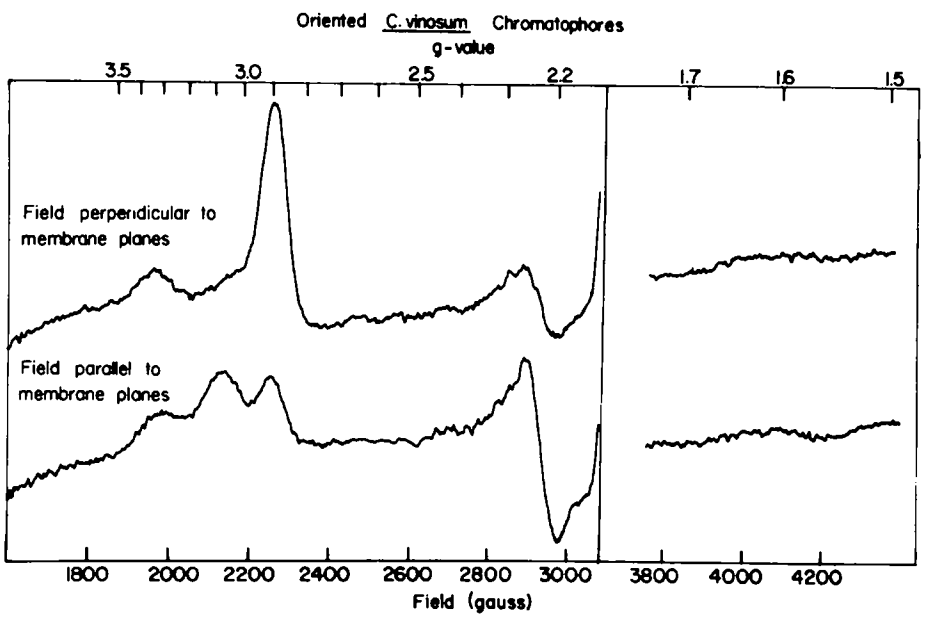

Fig. 9. EPR spectra of oriented chromatophore membranes. The upper spectrum was recorded with the applied magnetic field perpendicular to the plane of the membrane surfaces; the lower spectrum was recorded with the applied magnetic field parallel to the plane of the membrane surface. Spectrometer conditions are described in Fig. 3.

cytochrome *c*-553 and *c*-555 oxidized. Recording of EPR spectra with the normal to the membrane plane at various angles to the applied magnetic field shows that the cytochrome *c*-553 g_z absorption is maximal when the applied magnetic field is perpendicular to the membrane plane (Fig. 9), while the amplitudes of the cytochrome *c*-555 g_z and the cytochrome *c*-553 g_y and g_x absorptions become minimal. When the applied field is parallel to the membrane plane, the cytochrome *c*-553 g_z absorption becomes minimal, and the cytochrome *c*-555 g_z and the cytochrome *c*-553 g_y and g_x signals become maximal. These results suggest that the cytochrome *c*-553 and *c*-555 heme planes are perpendicular to each other. The cytochrome *c*-553 g_z component is found to lie perpendicular to the membrane plane, while the cytochrome *c*-555 g_z lies parallel to the membrane plane. If the chromatophores are not washed with ferricyanide, the cytochromes *c*-555 are found to be reduced in the oriented samples, and the orientation of the cytochrome *c*-553 hemes with respect to the membrane plane is found to be the same.

Magnetic studies on crystals of mammalian cytochrome *c* [31], and theoretical calculations of the g tensor for low spin hemes [32] indicate that the g_z component will be perpendicular to the heme plane. The results for the *C. vinosum* cytochromes *c*, suggest that the cytochrome *c*-553 heme planes lies nearly parallel to the membrane plane while the cytochrome *c*-555 heme plane lies perpendicular to the plane of the membrane surface.

Discussion

Relationship of the magnetic interactions with functional interactions between the c-cytochromes

The lack of a prominent magnetic interaction between the two cytochrome *c*-553 hemes and between the two cytochrome *c*-555 hemes is perhaps consistent with an absence of a prominent functional interaction. The redox state of one heme does not appear to affect the redox properties or room temperature oxidation kinetics of the companion, equipotential heme [2—10]. However, in this regard, it is interesting to note that with samples poised so that both cytochromes *c*-553 are reduced and the QFe is oxidized, illumination below 200 K illicits the rapid oxidation of either cytochrome *c*-553 heme and reduction of QFe [4], while continued illumination at 200 K further oxidizes only 20—30% of the second heme, generating the trapped I^-, suggesting that the oxidation of one heme has rendered the second less competent as an electron donor to $(BChl)_2^+$ at this temperature [16].

Another indication of possible interactions between the redox centers is provided by the variation in the position and lineshape of the g_z component of the EPR spectrum for the cytochrome *c*-553 hemes. This may reflect the existence of an electrostatic interaction between the two oxidized cytochrome *c*-553 hemes, or possibly between the single oxidized heme and the I^- or Q^-Fe. Mims and Peisach [33] have demonstrated for several low spin heme compounds that the presence of an electric field will cause a shift in the *g* values of the EPR spectrum, which will be observed most prominently at the g_z or g_x absorption. From their data it is possible to estimate that typically a point charge placed 12—20 Å away from a low spin heme will cause a 10

gauss shift in the g_z absorption assuming an effective dielectric constant of 3, and offer the possibility that the shift in the position of the cytochrome *c*-553 g_z peak observed in the various redox states may reflect an electrostatic interaction with the charges located on the I^-, Q^-Fe or accompanying oxidized cytochromes. Alternatively, however, the shift in the *g* value could be caused by a strain accompanying the oxidation of the cytochrome *c*-553, which could be translated into a protein conformational change near room temperature. Both of these effects will be expected to alter the electron affinity of the heme, and the possible relationship to functional activities are currently being investigated.

The observation of a prominent magnetic interaction between the cytochromes *c*-553 and *c*-555 surprisingly indicates that there exists a much closer association between the cytochrome *c*-553 and *c*-555 hemes than that which exists between these cytochromes and their oxidant $(BChl)_2$. The orthogonal arrangement and the close association between the cytochrome *c*-555 and *c*-553 hemes suggest that the edge of one heme may be as close as 3 Å from the plane of the other heme, perhaps with a side chain functioning as a ligand for the other heme. The proximity of the two hemes still leaves plausible two possibilities for the reported "switch" mechanism which ensures the apparent dominance of the cytochrome *c*-553 toward reduction of the $(BChl)_2^+$ [7—9]: the proximity could either provide a rapid electron equilibration between the cytochrome *c*-555 and *c*-553 hemes, or a direct heme-heme interaction. In the latter case such interactions could, upon reduction of the cytochrome *c*-553, alter the functional midpoint potential of the cytochrome *c*-555 such that its oxidation by $(BChl)_2^+$ becomes thermodynamically unfavorable.

Although currently there is no direct evidence for or against an electron equilibration between the two different cytochromes, the apparent close association supports the possibility that electron transfer can occur between the cytochrome *c*-553 and *c*-555 hemes. The orthogonal arrangement of these hemes might be expected to attenuate such an electron equilibration, however, it can be argued that the angular alignment of electron transfer groups will not be a critical element governing the occurrence or absence of an electron transfer reaction. For example, in a perhaps naive approach, the orbital overlap integral for atomic *p*-orbitals (molecular π-orbitals) separated by a distance goes to zero due to orbital symmetry only when the axes are perpendicular; variation of the angle by as little as 0.1 degree provides an overlap which is only about a factor of 10^3 smaller than the maximal orbital overlap which occurs when the axes of the orbitals are aligned parallel [34]. These considerations may suggest that in biological systems the angular arrangement of the electron transfer groups can provide, at most, a factor of 10^3 control over rates of reaction.

Instead, however, the rate of electron transfer might be expected to be critically determined by the redox potential differences between the donor and acceptor pairs [13,35]. The possible lack of an electron transfer between the cytochrome *c*-553 and *c*-555 hemes could be controlled by the functional midpoints on a transient basis, commensurate with the time scale of electron transfer, which may differ from the measured equilibrium redox midpoints as these are observed following molecular rearrangements. From this stand-

point the question of whether electron equilibration occurs between these hemes deserves reappraisal. The specificity of biological electron transfer could arise from this type of control of the functional midpoint by the interactions of the redox group with the surrounding protein environment, by the presence of electric fields, and by intermolecular protein couplings.

Summary of the structural interpretations

Fig. 10 summarizes the structural interpretations based upon the magnetic interactions described in this paper. The placement of the cytochrome *c*-555 hemes closer to the inner membrane interface than the cytochrome *c*-553 hemes has been suggested by the carotenoid shift data [1,36], and by the reduction of the cytochrome *c*-555 hemes by an agent located on the inner membrane-aqueous interface of the chromatophore [37]. The two hemes of each cytochrome pair are represented to have equivalent orientations with respect to the $(BChl)_2$, as may be expected from the equivalence in the oxidation kinetics. The close associations indicated between $(BChl)_2$ and I and between I and QFe may be expected from the magnetic exchange couplings observed between these pairs [16,38,39].

In contrast to the close association between the cytochrome *c*-553 and *c*-555 hemes, a relatively large separation is indicated between both the cytochromes *c*-553 and *c*-555 and their oxidant $(BChl)_2$, in which a minimum separation of 25 ± 3 Å between unpaired spins suggests that the minimum distance between the cytochrome heme edge and the $(BChl)_2$ edge is about 15 ± 3 Å. Predictions of the edge separation between these components have ranged from 8 Å [13] to 30—80 Å [11], based upon different descriptions of the electron tunnelling reaction. This relatively large separation suggests that a close association between donor and acceptor is not required for an efficient electron transfer. Similar separation between *c*-cytochromes and their physiological oxidant have been measured in other photosynthetic and respiratory systems [40—44]. In addition, if both the *C. vinosum* cytochromes *c*-555 and *c*-553 are oxidized directly by the $(BChl)_2^+$, then the results presented here may also tend to suggest that a specific orientation of the heme with respect to the

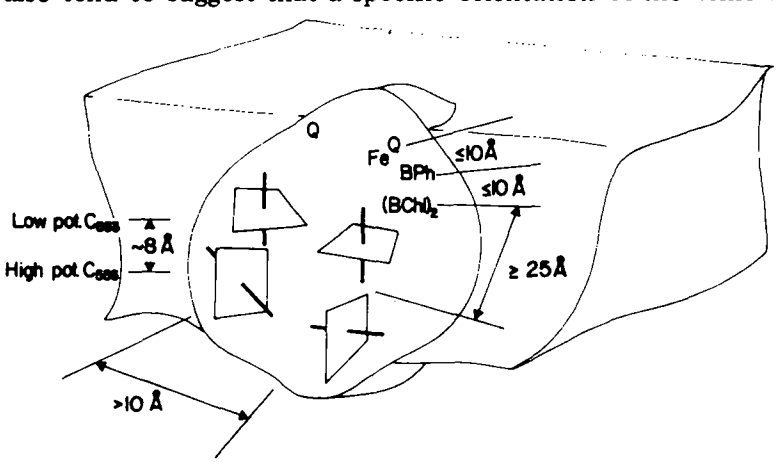

Fig. 10. Summary of the structural organization of the *C. vinosum* reaction center and the associated cytochromes *c*.

$(BChl)_2$ is not required for electron transfer. However, it is interesting to note that the equilibrium redox midpoints of these hemes differ by about 350 mV, and yet these cytochromes show similar oxidations rates. Clearly, more work needs to be done in order to understand the functional significance of the structural organization.

Acknowledgments

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